PATENT COOPERATION TREAT

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark
Office, PCT
2011 South Clark Place Room
CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 31 August 2001 (31.08.01)	
International application No. PCT/US00/40495	**Applicant's or agent's file reference** 3858PCT
International filing date (day/month/year) 27 July 2000 (27.07.00)	**Priority date** (day/month/year) 27 July 1999 (27.07.99)
Applicant BLUMENTHAL, Donald, K., II	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

13 February 2001 (13.02.01)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer Henrik NYBERG
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

Form PCT/IB/331 (July 1992)

US0040495

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 3858PCT	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/US00/40495	International filing date (day/month/year) 27 JULY 2000	Priority date (day/month/year) 27 JULY 1999
International Patent Classification (IPC) or national classification and IPC IPC(7): G01N 33/53 and US Cl.: 435/4, 7.72, 15.21; 436/86, 89, 172		
Applicant UNIVERSITY OF UTAH RESEARCH FOUNDATION		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 6 sheets.

   ☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 0 sheets.

3. This report contains indications relating to the following items:

   I ☒ Basis of the report

   II ☐ Priority

   III ☐ Non-establishment of report with regard to novelty, inventive step or industrial applicability

   IV ☐ Lack of unity of invention

   V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

   VI ☐ Certain documents cited

   VII ☐ Certain defects in the international application

   VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
13 FEBRUARY 2001	19 MARCH 2002
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer ARLEN SODERQUIST Telephone No. (703) 308-3989

INTERNATIONAL PRE[redacted]INARY EXAMINATION REPORT

International application No.
[redacted]/US00/40495

I. Basis of the report

1. With regard to the **elements** of the international application: *

[X] the international application as originally filed

[X] the description:
pages 1-19 , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

[X] the claims:
pages 20-26 , as originally filed
pages NONE , as amended (together with any statement) under Article 19
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

[X] the drawings:
pages 1-7 , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

[X] the sequence listing part of the description:
pages NONE , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

[ ] the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

[ ] the language of publication of the international application (under Rule 48.3(b)).

[ ] the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

[X] contained in the international application in printed form.

[ ] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[X] furnished subsequently to this Authority in computer readable form.

[ ] The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[X] The statement that the information recorded in computer readable form is identical to the writen sequence listing has been furnished.

4. [X] The amendments have resulted in the cancellation of:

[X] the description, pages NONE

[X] the claims, Nos. NONE

[X] the drawings, sheets/~~fig~~ NONE

5. [ ] This report has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

** Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

***Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.*

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. [redacted]/US00/40495

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. statement

Novelty (N)	Claims	10,14,16-20,22-27	YES
	Claims	1-9,11-13,15,21	NO
Inventive Step (IS)	Claims	NONE	YES
	Claims	1-27	NO
Industrial Applicability (IA)	Claims	1-27	YES
	Claims	NONE	NO

2. citations and explanations (Rule 70.7)

Claims 1-9, 13, 15 and 21 lack novelty under PCT Article 33(2) as being anticipated by Lee et al. In the paper Lee et al teaches a new approach to assay endo-type carbohydrases using bifluorescent-labeled substrates for glycoamidases and ceramide glycanases. Glycoamidases and ceramide glycanases are important "endo-type" enzymes for structural elucidations of glycoconjugates as well as for construction of neoglycoconjugates. The assay methods currently available for these enzymes are tedious and do not permit continual assay of the enzyme activities. The authors modified a desialylated biantennary glycopeptide with 2-naphthylacetic acid at the N-terminus and at the nonreducing terminal galactosyl residues with mono-N-dansylethylenediamine, via a specific oxidation of the C-6 hydroxyl group with galactose oxidase. see figure 1 for the two substrates used. In such a substrate, the naphthyl fluorescence (λ_{em} = 335 nm) is quenched due to absorption of its emitted light by the dansyl group, which in turn results in emission of fluorescence (λ_{ex} = 520 nm) by the latter. However, when the link between the two fluorophores is severed (a covalent modification) by glycoamidase (PNGase), the energy transfer ceases to occur. Consequently the emission of the dansyl fluorescence and the quenching of naphthyl fluorescence diminish or disappear. Likewise, the energy transfer between the fluorophores in an alkyl lactoside containing a dansyl group at the terminal position of aglycon and a 2-naphthylmethyl group on the galactosyl residue is also eliminated by the glycosidic cleavage by a ceramide glycanase from American leech, Macrobdella decora, resulting in enhancement of the naphthyl emission and decrease in the dansyl emission. The substrates presented here permit continuous fluorescent monitoring of the enzymic reaction. This allows precise analyses of enzyme kinetics not possible with the conventional assay methods for the endo-type enzymes which usually require separation of reaction products.

Claims 1-9, 5, 7, 13, 15 and 21 lack novelty under PCT Article 33(2) as being anticipated by Zhang et al. In the paper Zhang et al presents a fluorogenic substrate for measuring -amylase (E.C. 3.2.1.1.) activity was prepared by double labeling soluble starch (Continued on Supplemental Sheet.)

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

V. 2. REASONED STATEMENTS - CITATIONS AND EXPLANATIONS (Continued):
with 5-(4,6-dichlorotrizain-2-yl)aminofluorescein and Procion Red MX8B. Because the absorption spectrum of Procion Red MX8B overlaps the fluorescein emission spectrum, Procion Red efficiently quenches fluorescein emission when it is closer than the critical radius for fluorescence energy transfer. When amylase catalyzes cleavage (a covalent modification) of a starch molecule between a fluorescein and a Procion Red MX8B, the distance between the two labels increases and the degree of quenching decreases. The rate at which the fluorescence intensity increases is proportional to amylase activity. To maximize the sensitivity it is critical to maximize the amount of Procion Red MX 8B coupled to the starch and to use a high-precision spectrofluorimeter which can measure a small rate of increase in fluorescence above a large constant background.

Claims 1-5, 7-9, 13, 15 and 21 lack novelty under PCT Article 33(2) as being anticipated by Meldal et al. In the paper Meldal et al teaches anthranilamide and nitrotyrosine as a donor-acceptor pair in internally quenched fluorescent substrates for endopeptidases and multicolumn peptide synthesis of enzyme substrates for subtilisin Carlsberg and pepsin. The preparations of N-Fmoc-3-nitro-L-tyrosine and N-Boc-anthranilic acid Dhbt ester (Fmoc = fluoren-9-ylmethyloxycarbonyl; Boc = tert-butyloxycarbonyl; Dhbt = 3,4-dihydro-4-oxo-1,2,3-benzotriazo-3-yl) and their application to parallel multiple column solid-phase peptide synthesis is described. A series of peptide substrates (Table 1) containing an anthraniloyl group at the N-terminus and a 3-nitrotyrosyl residue close to the C-terminus were synthesized. The fluorescence of the anthraniloyl group, intramolecularly quenched by the 3-nitrotyrosine, increased with cleavage of peptide bonds situated between the 2 groups. The quenching mechanism was of the long-range resonance energy transfer type and long peptide substrates were constructed and used for kinetic measurements of subtilisin Carlsberg and pepsin. Complete quenching was observed even with > 20 between the centers of the chromophores, and substrates with 50 between the chromophores were synthesized. The importance of long substrates for optimal enzymic activity was demonstrated.

Claims 1, 3, 5, 7, 13, 15 and 21 lack novelty under PCT Article 33(2) as being anticipated by Taliani et al. In the paper Taliani et al present a continuous assay of hepatitis C virus protease based on resonance energy transfer depsipeptide substrates. Hepatitis C virus (HCV) is the major causative agent of non-A non-B hepatitis, an important health problem with an estimated 50 million people infected worldwide. Among the possible targets for therapeutic intervention, the serine protease contained within the N-terminal region of non-structural protein 3 (NS3 protease) is so far the best characterized. In vitro characterization of synthetic substrates based on all the natural cleavage sites (as well as a series of analogs) has consistently revealed poor kinetic parameters, making them unsuitable for sensitive high-throughput screening. To overcome these difficulties, we have recently developed depsipeptide substrates incorporating an ester bond between residues P_1 and P_{-1} (figure 1). Due to ready transesterification of the scissile bond to the acyl-enzyme intermediate, these substrates showed very high kcat/Km values, enabling detection of activity with subnanomolar NS3 concentrations. We have used the same principle to synthesize internally quenched depsipeptide fluorogenic substrates based on resonance energy transfer between the donor/acceptor couple 5-[(2'-aminoethyl)amino]-naphthalene sulfonic acid/4-[[4'-(dimethylamino)phenyl]azo]benzoic acid, and developed a continuous assay for NS3 activity. Substrate cleavage is linear with enzyme concentration: depending on the conditions chosen, they estimated a detection limit for NS3 between 1 nM and 250 pM. The suitability of the assay for evaluation of inhibitors was established using as competitor a tridecapeptide corresponding to the natural NS4A/4B cleavage site; this gave an IC50 of 30 microM, well in agreement with the previously found Km value (40 microM).

Claims 1, 3-5, 7, 12-13, 15 and 21 lack novelty under PCT Article 33(2) as being anticipated by Zandonella et al. In the paper Zandonella et al teach fluorogenic alkyldiacyl glycerols as substrates for the determination of lipase activity and stereoselectivity. They synthesized enantiomeric alkyldiacyl glycerols containing pyrene as a fluorophore, and the trinitrophenylamino residue as a fluorescence quencher, both covalently bound to the -end of the respective acyl chains. Fluorescence is efficiently quenched due to resonance energy transfer in the intact molecules. Chemical or enzymic release of the fatty acyl chains led to fluorescence dequenching. From the time-dependent increase in fluorescence intensity lipase activity and stereoselectivity can be determined, if enantiomerically pure substrates are used.

Claims 1-7, 9, 11, 13, 15 and 21 lack novelty under PCT Article 33(2) as being anticipated by Hirano et al. In the published application Hirano et al teaches determination of double-stranded nucleic acids-cleaving enzyme activities by fluorescence resonance energy transfer (FRET) analysis. Described is a method to determine the double-stranded nucleic acid enzyme activity by FRET, where a nucleic acid capable of forming intermolecular duplex labeled with an energy donor (e.g. fluorescein) and an energy acceptor (e.g. rhodamine X) at both ends, respectively, is used as a substrate. The increase of fluorescence resulting from the enzymic digestion of the nucleic acid substrate can be observed by fluorometry The method was demonstrated by digestion with restriction endonucleases HindIII and PvuII and their resp. substrates. See the figures for the manner in which the method works.

Claims 1-27 lack an inventive step under PCT Article 33(3) as being obvious over Macala et al, Shultz et al or Ventura et al

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

in view of Blumenthal, Mathies et al (US Patent 5,654,419) and Hirano et al, Lee et al, Meldal et al, Taliani et al, Zandonella et al or Zhang et al (last six references as explained above).

In the paper Macala et al teaches measurement of cAMP-dependent protein kinase activity using a fluorescent-labeled Kemptide. Traditional protein kinase assays include the use of ^{32}P-labeled ATP as phosphate donor and a substrate protein or peptide as phosphoreceptor. Since this approach has a number of drawbacks in addition to generating ionizing radiation, several non-isotopic methods have been developed. Although shown to reflect the activity of purified enzymes, none have been demonstrated to detect physiological changes in endogenous enzyme activity in cell homogenates. Here, studies were performed to examine the kinetics, reproducibility, and optimal assay conditions of a novel non-radioisotopic kinase assay that detects protein kinase A (PKA) activity by phosphorylation of the peptide substrate, Kemptide, covalently bound to a fluorescent molecule (fluorescamine-labeled Kemptide; f-Kemptide). Fluorescence was determined by spectrofluorometry with excitation at 568 nm and emission at 592 nm. Basal and agonist-induced PKA activities in epithelial cell homogenates were measured. The kinetics of f-Kemptide were similar to the standard radioisotopic method with intra-assay and inter-assay variations of 5.6 $\pm$ 0.8% and 14.3 $\pm$ 2.6%, respectively. Neither fluorescence quenching nor enhancing effects were found with consistent amounts of homogenate protein. Specific PKA activity was determined as the IP20-inhibitable fraction to account for nonspecific phosphorylation, perhaps due to S6 kinase or a similar enzyme. The basal activity of 38% of total PKA in A6 cells increased by 84% after exposure to vasopressin and by 58% after short exposure to forskolin. In T84 cells exposed to VIP there was a 360% increase over basal activity. Thus, these results show that f-Kemptide exhibits acceptable kinetics, and that the assay system can quantitatively and reproducibly measure basal and stimulated PKA activity in cell homogenates. Macala et al does not teach the substrate having two dyes attached or a library of compounds.

In the patent Shultz et al teaches a non-radioactive enzyme assay. The invention is directed to the assay and purification of proteins, and particularly to the non-radioactive assay and purification of protein kinases, phosphatases and protease by incubating the enzyme with a substrate modified peptide to form a product modified peptide under conditions where the enzyme is active. The product modified peptide and substrate modified peptide are then separated, and the product modified peptide is measured. The invention is also directed to kits and bioreagents for performing the assays. In the patent table 1 shows a list of the substrates which have a fluorescent dye attached to the substrate. Shultz et al does not teach the substrate having two dyes attached or a library of compounds.

In the paper Ventura et al teaches phorbol ester regulation of opioid peptide gene expression in myocardial cells. Role of nuclear protein kinase C. Opioid peptide gene expression was characterized in adult rat ventricular cardiac myocytes that had been cultured in the absence or the presence of phorbol 12-myristate 13-acetate. The phorbol ester induced a concentration- and time-dependent increase of prodynorphin mRNA, the maximal effect being reached after 4 hours of treatment. The increase in mRNA expression was suppressed by incubation of cardiomyocytes with staurosporine, a putative protein kinase C inhibitor, and was not observed when the cells were cultured in the presence of the inactive phorbol ester 4α-phorbol 12,13-didecanoate. Incubation of cardiac myocytes with phorbol 12-myristate 13-acetate also elicited a specific and staurosporine-sensitive increase in immunoreactive dynorphin B, a biologically active end product of the precursor, both in the myocardial cells and in the culture medium. In vitro run-off transcription assays indicated that transcription of the prodynorphin gene was increased both in nuclei isolated from phorbol ester-treated myocytes and in nuclei isolated from control cells and then exposed to phorbol 12-myristate 13-acetate. No transcriptional effect was observed when cardiac myocytes or isolated nuclei where exposed to 4α -phorbol 12,13-didecanoate. The phorbol ester-induced increase in prodynorphin gene transcription was prevented by pretreatment of myocytes or isolated nuclei with staurosporine, suggesting that myocardial opioid gene expression may be regulated by nuclear protein kinase C. In this regard, cardiac myocytes expressed protein kinase C-α, -δ, -ε, and -ζ, as shown by immunoblotting. Only protein kinase C-α and protein kinase C-ε were expressed in nuclei that have been isolated from control myocytes, suggesting that these 2 isotypes of the enzyme may be part of the signal transduction pathway involved in the effect elicited by the phorbol ester an opioid gene transcription in isolated nuclei. The incubation of myocardial nuclei isolated from control cells in the presence of a protein kinase C activator induced the phosphorylation of the myristylated alanine-rich protein kinase C substrate peptide, a specific fluorescent substrate of the enzyme. The possibility that prodynorphin gene expression may control the heart function through autocrine or paracrine mechanisms is discussed. Ventura et al does not teach the substrate having two dyes attached or a library of compounds.

In the paper Blumenthal reviews the development and characterization of fluorescently-labeled myosin light chain kinase calmodulin-binding domain peptides. In the review, the development and characterization of peptides based on the sequence of the calmodulin-binding domain of skeletal muscle myosin light-chain kinase which were labeled with the fluorescent reagent, acrylodan is described. The use of these fluorescently-labeled peptides to study various aspects of calmodulin-peptide interactions including binding affinity, stoichiometry, specificity, changes in peptide conformation, and thermal stability of the peptide-calmodulin complex is demonstrated. Page 46 discusses the formation of analogs by replacing different amino acids within a natural sequence to examine these properties. Blumenthal also teaches the formation of a library peptides with different fluorescent labels. Page 46 also discusses the change in the acrodan fluorophore as the environment changes and how this is useful in determining various properties. The peptides exhibit many of the salient features seen with calmodulin-target enzyme interactions. The fluorescently-labeled peptides should serve as useful models for

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

studying calmodulin-target enzyme interactions at the molecular level.

In the patent Mathies et al discusses fluorescent labels and their use in separations. Fluorescent labels having at least one donor and at least one acceptor fluorophore bonded to a polymeric backbone in energy transfer relationship, as well as methods for their use, are provided. Of particular interest are the subject labels wherein the polymeric backbone is a nucleic acid and the donor fluorophore is bonded to the 5' terminus of said nucleic acid. The range of distances between donor and acceptor fluorophores is chosen to ensure efficient energy transfer, and can be modulated to affect the label mobility. Such labels find use as primers in applications involving nucleic acid chain extension, such as sequencing, PCR and the like. Sequencing primers labeled with two fluorophores were constructed and their fluorescent properties optimized. Dual fluorophore-labeled PCR primers for sizing of short tandem repeats (STRs) in the tyrosine hydroxylase, thrombopoietin, cytostatic factor, and von Willebrand factor genes were also prepared. These primers were used to amplify the STRs and the amplicons were separated by capillary gel electrophoresis. Column 4 gives many examples of the types of dyes that may be used as the donor and acceptor pairs.

It would have been obvious to one of skill in the art to have incorporated a double label selected from those taught by Mathies et al, Hirano et al, Lee et al, Meldal et al, Taliani et al, Zandonella et al or Zhang et al in the Macala et al, Shultz et al or Ventura et al substrates because of the ability to detect changes in the substrate due to covalent modifications of the substrates as shown by Hirano et al, Lee et al, Meldal et al, Taliani et al, Zandonella et al or Zhang et al and the ability to carry out a continuous monitor without separation. One of skill in the art would also have recognized that libraries of substrates as taught by Blumenthal would have allowed the Macala et al, Shultz et al or Ventura et al substrates to be used for characterizing enzyme properties as shown by Blumenthal.

Claims 1-27 meet the criteria set out in PCT Article 33(4), because they clearly would be usable to monitor or determine properties of enzymes.

---------- NEW CITATIONS ----------

D. K. Blumenthal "Development and Characterization of Fluorescently-Labeled Myosin Light Chain Kinase Cadmodulin-Binding Domain Peptides" Molecular and Cellular Biochemistry, 1993, Vol. 127/128, pages 45-50, see entire document.

JP 11-56398 A (HIRANO et al) 03 March 1999, see English abstract and figures.

L. J. Macala et al, "Measurement of cAMP-Dependent Protein Kinase Activity Using a Fluorescent-Labeled Kemptide" Kidney International 1998, Vol 54, pages 1746-1750, see entire document.

M. Meldal et al, "Anthranilamide and Nitrotyrosine as a Donor-Acceptor Pair in Internally Quenched Fluorescent Substrates for Endopeptidases: Multicolumn Peptide Synthesis of Enzyme Substrates for Subtilisin Carlsberg and Pepsin" Analytical Biochemistry 1991, Vol. 195, pages 141-147, see entire document.

US 5,580,747 A (SHULTZ et al) 03 December 1996, see entire document.

W. Stocker et al, "Fluorescent Oligopeptide substrates for Kinetic Characterization of the Specificity of Astacus Protease" Biochemistry 1990, Vol. 29, pages 10418-10425, see entire document.

M. Taliani et al, "A Continuous Assay of Hepatitis C Virus Protease Based on Resonance Energy Transfer Depsipeptide Substrates" Analytical Biochemistry 1996, Vol. 240, pages 60-67, see entire document.

C. Ventura et al, "Phorbol Ester Regulation of Opioid Peptide Gene Expression in Myocardial Cells" The Journal of Biological Chemistry 15 December 1995, Vol. 270, No. 50, pages 30115-30120, see entire document.

C. Zandonella et al, "Fluorogenic Alkyldiacyl Glycerols as Substrates for the Determination of Lipase Activity and Sterioselectivity" Journal of Fluorescence 1997, Vol. 7, No. 1 (supplement), pages 185S-186S, see entire document.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(72) Inventor; and

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(74) Agents: **BOND, Laurence, B.** et al.; Trask Britt, P.O. Box 2550, Salt Lake City, UT 84110 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

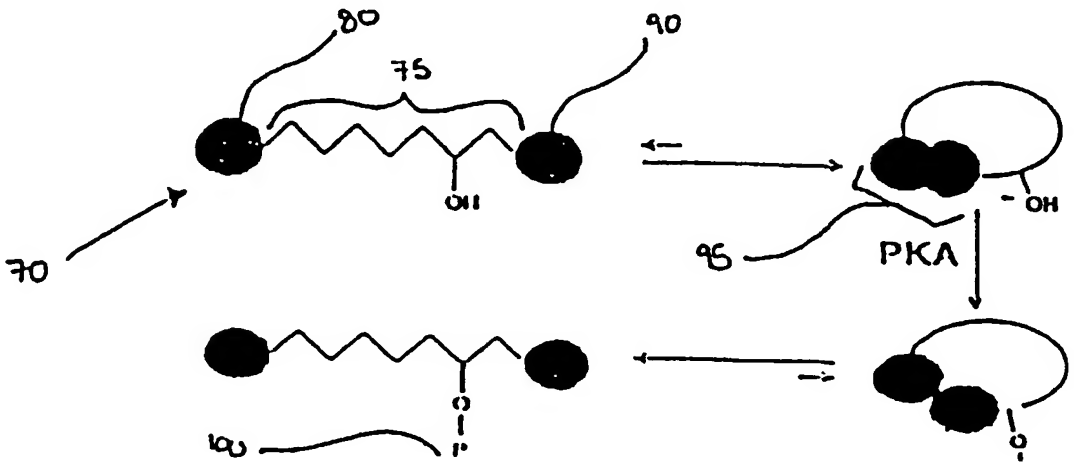
(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

(88) Date of publication of the international search report:
16 August 2001

[Continued on next page]

(54) Title: HOMOGENEOUS FLUORESCENCE METHOD FOR ASSAYING STRUCTURAL MODIFICATIONS OF BIOMOLECULES



(57) Abstract: Double-labeled protein biomolecular substrates (70) and methods for the homogenous assay of processes which include covalent modification of the substrates (70) to form a detectable species are described. The biomolecular substrates (70) of the instant invention are labeled at two positions (80, 90) with two fluorescent dyes or with a fluorescent dye and a nonfluorescent dye. The two labeling dyes of the unmodified substrate (70) stack (95), thereby quenching the substrate's fluorescence. Upon covalent modification of the double-labeled substrate (70), however, the intramolecularly stacked dyes (95) dissociate and the fluorescence changes markedly. Examples are described for the preparation and use of substrates (70) for phosphorylation assays. Methods of invention do not require separation of the modified and unmodified substrates (70), nor do they require other special reagents or radioactive materials. Therefore the substrates can be used for monitoring intracellular processes of living cells.

WO 01/07638 A3

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/40495

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : G01N 33/53
US CL : 435/4, 7.72, 15.21; 436/86, 89, 172

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/4, 7.72, 15.21; 436/86, 89, 172

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X - Y	K. B. Lee et al, "A New Approach to Assay Endo-Type Carbohydrases: Bifluorescent-Labeled Substrates for Glycoamidases and Ceramide Glycanases" Analytical Biochemistry, 01 September 1995, Vol. 230, No. 1, pages 31-36, see entire document.	1-2,5-7,13, 15,21 ---------- 3-4,8-12,14,16-20,22-27
X - Y	K. Matsuoka et al, "A Bi-Fluorescence-Labeled Substrate for Ceramide Glycanase Based on Fluorescence Energy Transfer" Carbohydrate Research, 16 October 1995, Vol. 276, No. 1, pages 31-42, see entire document.	1,2,5-7,13,15,21 ---------- 3,4,8-12,14,16-20,22-27

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents.	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 16 JANUARY 2001	Date of mailing of the international search report 23 FEB 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer ARLEN SODERQUIST Telephone No. (703) 308-3989

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International application No.
PCT/US00/40495

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B. FIELDS SEARCHED
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CA, BIOSIS, and MEDLINE files in STN
search terms: kinase, fluoresc?, label?, absor?, resonant?, energy, transfer?, Exchang?, covalent?, double, di, bi, two, label, fluoroph?, modif?, bind?, bond?, complex?, bound?, immobil?